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Journal of Virology

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J. Virol. 1997, 71(2):1692.

JOURNAL OF VIROLOGY, Feb. 1997, p. 1692–1696 Vol. 71, No. 2
0022-538X/97/$04.00+0

Inhibition of Human Immunodeficiency Virus Fusion by a Monoclonal Antibody to a Coreceptor (CXCR4) Is both Cell Type and Virus Strain Dependent

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Received 12 July 1996/Accepted 8 November 1996

CXCR4 (also termed fusin, LESTR, or HUMSTR) is a member of the G-protein-coupled chemokine receptor family with seven membrane-spanning domains. CXCR4 acts as a coreceptor for syncytium-inducing human immunodeficiency virus type 1 (HIV-1) strains, conferring entry into CD4$^+$ cells. We show here that a novel mouse monoclonal antibody (12G5) that recognizes CXCR4 blocked cell-to-cell fusion and cell free-virus infection of CXCR4$^+$ CD4$^+$ RD rhabdomyosarcoma cells by seven HIV-1 and HIV-2 strains that had various cell tropisms for different CD4$^+$ human cell types. Yet the majority of the members of the same virus panel resisted 12G5 inhibition on T-cell lines. When inhibition was observed on these cell types, it was both cell type and virus strain dependent. In at least one situation, 12G5 failed to block LAI infection of cells expressing CXCR4 as the only available coreceptor. Our observations suggest that CXCR4 could be processed or presented differently depending on the cell type, allowing some strains to evade 12G5 inhibition. Alternatively, since several of the viruses could infect certain CXCR4$^-$ CD4$^+$ cell lines, it is conceivable that alternative coreceptors are active, enabling individual HIV strains to choose between compatible coreceptors during entry into cells. Moreover, the strain dependency of 12G5 inhibition implies that the interaction of different HIVs with CXCR4 varies.

Human immunodeficiency virus (HIV) enters cells by attaching to cell surface CD4 before interacting with a coreceptor that triggers fusion of viral and cellular membranes. CXCR4 (also called fusin, LESTR, or HUMSTR) (14, 16, 17, 19, 26) is a member of the seven-transmembrane-domain, G-protein-coupled receptor family and has been shown to act as a coreceptor for syncytium-inducing, T-cell-line-adapted HIV type 1 (HIV-1) strains (15). Other chemokine receptors can also be used as coreceptors by HIV-1 strains. CCR5 (30) acts as a coreceptor for non-syncytium-inducing (NSI) macrophage-tropic strains (1, 10, 12), while some primary strains can also use CCR3 (5, 11, 32). Other chemokine receptors, e.g., CCR2b, have been shown to be functional at least for cell-to-cell fusion (11).

Chemokines are small (8- to 10-kDa) proteins and are the natural ligands for chemokine receptors. For instance, the chemokines RANTES, MIP-1α, and MIP-1β all interact with CCR5 (30), and stroma-derived factor (SDF) has recently been shown to be a ligand for CXCR4 (2, 27). These chemokines block infection of HIV-1 strains that use CCR5 and CXCR4, respectively. For example, RANTES, MIP-1α, and MIP-1β inhibit infection of CCR5-expressing peripheral blood mononuclear cells (PBMCs) by NSI macrophage-tropic viruses, while SDF has been shown to block infection of CXCR4$^+$ HeLa/CD4 cells by the T-cell-line-adapted HIV-1 strain LAI. Here, we show that a monoclonal antibody (MAb) specific for CXCR4 also blocks HIV infection of CXCR4$^+$ cells, although inhibition is cell type and virus strain specific.

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12G5 is a recently described mouse MAb that specifically recognizes CXCR4 but not other members of the chemokine receptor family, including CCR1-5 and interleukin-8 receptors α and β (13). As far as we know, 12G5 is the only MAb available that recognizes the HIV coreceptor CXCR4. Figure 1A shows the levels of cell surface CXCR4 expression determined by flow cytometry after 12G5 staining of the CD4$^+$ T-cell lines MOLT-4 and C8166, the CD4-transfected human rhabdomyosarcoma cell line RD, and the human glioma cell line U87 as well as primary macrophages purified by adherence to plastic and cultured for 1 or 5 days (22, 31). Flow cytometry was carried out as previously described (21). HIV infectivity of macrophages or PBMCs is routinely estimated by infecting cells that have been cultured for 5 days after purification from blood or buffy coats. We were therefore particularly interested in assessing CXCR4 expression for cultures of this age. Prior to staining with 12G5, macrophages and PBMCs were preincubated with 5% heat-aggregated hyperimmune gamma globulin (Miles Cutler) on ice for 30 min to block any nonspecific binding to Fc receptors. Figures 1B and C show the results of reverse transcription-PCR (RT-PCR) and Northern blot analyses, respectively, of RNA prepared from the same cell types as shown in Fig. 1A. RNA for RT-PCR was prepared with RNAzol. cDNA was then prepared from 5 μg of RNA by using the Stratagene RT-PCR kit. One-twentieth of the cDNA prepared was included in the PCR reactions. PCR for CXCR4 used the primers 5′-TAG ATA TCT TAC CAT GGA GGG GAT CAG-3′ and 5′-TAG CGG CGC TTA GTG GAG TGA AAA CTT G-3′, corresponding to the positive and negative strands, respectively, and amplifying a 1,044-bp fragment. The positive-strand primer incorporated a 5′ tail which encoded an *Eco*RV site, and the minus strand incorporated a 3′ tail encoding a

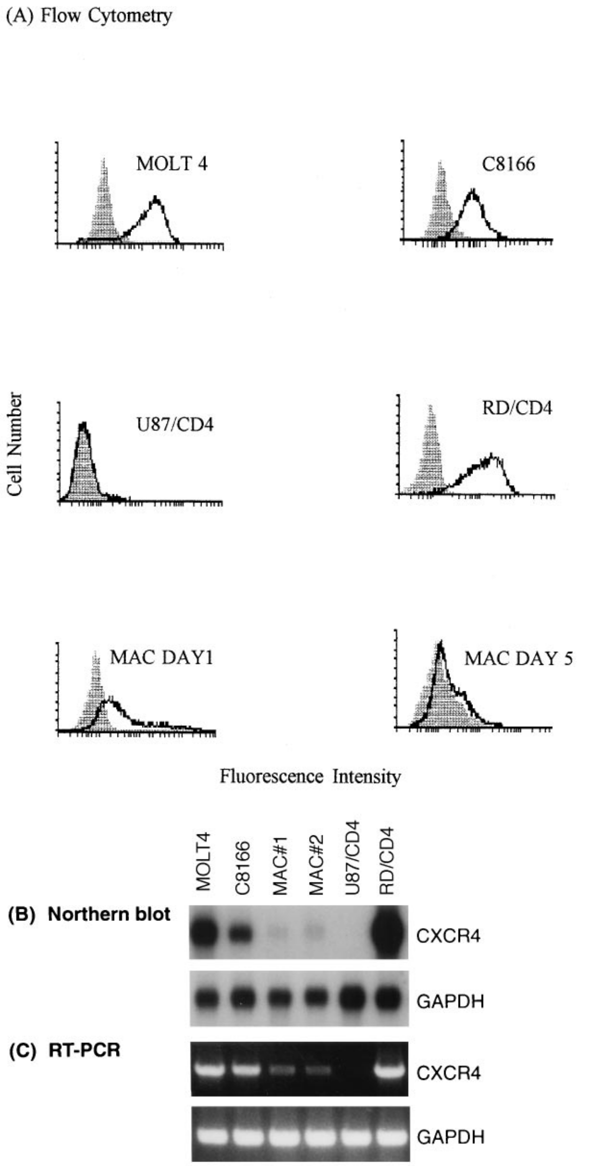

FIG. 1. Detection of CXCR4 cell surface glycoprotein and mRNA. (A) Fluorescence-activated cell sorter analysis of cell surface CXCR4 expression by 12G5 staining on MOLT-4, C8166, U87/CD4, and RD/CD4 cells as well as on primary human macrophages (MAC) cultured for 1 and 5 days. (B and C) CXCR4 mRNA detection by Northern blotting (B) and by RT-PCR (C) on the same cells as in panel A, except two batches of primary macrophages (cultured for 5 days) were included for each method.

*Not*I site. Conditions for CXCR4 amplification were 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences were amplified by using the positive-strand primers 5′-TGG ATA TTG CCA TCA ATG ACC-3′ and the negative-strand primer 5′-GAT GGC ATG GAC TGT GGT CAT G-3′. Conditions for the PCR were 40 cycles of 95°C for 30 s, 65°C for 1 min, and 72°C for 30 s. Control PCR analysis of the RNA preparations was consistently negative for both CXCR4 and GAPDH DNA. mRNA for Northern blot analysis was prepared from 10^6 to 10^7 cells (Pharmacia Quickprep Micro mRNA isolation system), and 1 μg was fractionated on a 1.2% agarose–formaldehyde gel. RNA was transferred overnight onto a Genescreen Plus membrane (NEN) with 10× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA) and then baked at 80°C for 2 h. ^{32}P-labelled CXCR4 and GAPDH double-stranded probes were prepared by random priming (Amersham) and hybridized to the membrane in Quickhyb solution (Stratagene) for 1 h at 65°C. The membrane was washed twice for 15 min at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and once for 30 min at 65°C in 0.1× SSC–0.1% sodium dodecyl sulfate. The blot was then exposed at −70°C for 1 week. Cell lines used in Fig. 1 and throughout this study, including RD/CD4, U87/CD4, HeLa/CD4, SCL/CD4, Daudi, and $CD4^+$ T-cell lines C8166, MOLT-4, MT-2, SSCEM, and Sup T1, have all been described previously (4, 6–8, 18, 20, 23, 24). WI-38/t cells are simian virus 40-transformed counterparts of the WI-38 human diploid cell line (28).

Figure 1 shows that 12G5 detection of cell surface CXCR4 by flow cytometry correlated well with CXCR4 mRNA detection by RT-PCR or by Northern blotting. Both $CD4^+$ T-cell lines (MOLT-4 and C8166) as well as RD cells were highly positive for CXCR4 cell surface expression as well as for CXCR4 mRNA expression by RT-PCR and by Northern blotting. Neither CXCR4 protein nor mRNA could be detected in U87 cells. Primary macrophages were highly positive for cell surface CXCR4 expression after 1 day of culture; however, expression had dropped substantially by 5 days. Northern blot and RT-PCR analyses of 5-day-old primary macrophages were positive for CXCR4 mRNA but at a lower level than cell lines MOLT-4, C8166, and RD.

Table 1 summarizes the data shown in Fig. 1 and includes a survey of other cell types, including Daudi (a B-cell line), HeLa/CD4, and the $CD4^+$ T-cell lines H9 and MT-2 as well as phytohemagglutinin- and interleukin-2-stimulated PBMCs cultured for 5 days as described previously (32). All of these cell types stained positive for CXCR4 cell surface expression. Expression of CXCR4 by cell lines ranged from 35.5% for HeLa/

TABLE 1. Correlation of CXCR4 expression and infection by T-cell-line-passaged HIV-1

Cell type	Source	Level of CXCR4 expression determined by:		Infection by HIV-1 LAI
		mRNA[a]	12G5[b]	
RD/CD4	Rhabdomyosarcoma	++	93.3	+
HeLa/CD4	Cervical carcinoma	++	35.5	+
U87/CD4	Glioma	−	<2	−
SCL/CD4	Skin	−	<2	−
WI-38t/CD4	Lung	−	<2	−
Daudi/CD4	B cell	++	88.4	+
C8166	T cell	++	72.0	+
MOLT-4	T cell	++	89.5	+
Sup T1	T cell	NT[c]	81.5	+
H9	T cell	NT	51.4	+
MT-2	T cell	NT	99.0	+
PBMC[d]		++	27.8	+
Macrophage[d]		+	5.2	(−)[e]

[a] CXCR4 mRNA was measured by RT-PCR. −, no CXCR4 expressed; +, weak CXCR4 expression; ++, high-level CXCR4 expression.
[b] Values are percentages of cells expressing CXCR4 as determined by fluorescence-activated cell sorter analysis of 12G5 staining.
[c] NT, not tested.
[d] CXCR4 expression was estimated after 5 days of culturing PBMCs or macrophages. The percentages of 12G5-positive cells are averages derived from staining 10 batches of PBMCs and 3 batches of macrophages, each from different donors.
[e] (−), inefficient infection only.

TABLE 2. Summary of expression of CXCR4 and susceptibility to infection by HIV-1 and HIV-2 strains

CD4+ cell line or type used	Level of CXCR4 expression[a]	Infection by[b]: HIV-1 strain				HIV-2 strain	
		LAI	RF	Gun-1wt	Gun-1var	ROD	CBL-23
RD/CD4	++	+	+	+	+	+	+
HeLa/CD4	++	+	+	+	+	+	+
U87/CD4	−	−	−	−	+	+	+
WI-38/t/CD4	−	−	−	−	+	+	NT[c]
CD4+ T-cell lines	++	+	+	+	+	+	+
PBMC	++	+	+	+	+	+	+
Macrophage	+	(−)[d]	(−)	+	(−)	+	NT

[a] −, no CXCR4 expression; +, low-level CXCR4 expression; ++, high-level CXCR4 expression.
[b] −, not susceptible; +, susceptible.
[c] NT, not tested.
[d] (−), inefficient infection only.

CD4 to >99% for MT-2 cells. Other human cell lines, such as SCL skin and WI-38/t lung cells, were negative for CXCR4 expression by 12G5 staining and for CXCR4 mRNA, like U87 glioma cells. Thus, CXCR4 is expressed widely on human hematopoietic cell types and on some nonlymphoid cell types but is absent from the surfaces of human U87 glioma, SCL skin, and WI-38/t lung cells and is only weakly expressed on 5-day-old macrophages derived from blood monocytes. This pattern of CXCR4 expression correlates well with the sensitivity of these $CD4^+$ cell types to infection by T-cell-line-passaged HIV-1 strains such as LAI and RF (Tables 1 and 2) (4, 6). However, HIV-2 strains (e.g., ROD) and some HIV-1 strains (e.g., the GUN-1 variant [GUN-1var]) show a broader tropism and can infect several $CXCR4^-$ cell types (e.g., U87/CD4) (6, 20, 22, 31, 33).

We tested whether 12G5 could inhibit cell-to-cell fusion induced by a panel of HIV-1 and HIV-2 strains. The HIV-1 and HIV-2 strains tested were chosen because they show distinct tropisms for various $CD4^+$ cell types (summarized in Table 2), but all infect $CD4^+$ T-cell lines as well as HeLa/CD4 and RD/CD4 cells. The wild-type GUN-1 strain of HIV-1 (GUN-1wt) is dual tropic and, unlike LAI and RF, efficiently infects primary macrophages as well as $CD4^+$ T-cell lines (22, 31). A single amino acid change in the V3 loop of GUN-1var results in the loss of macrophage tropism but confers efficient infection of $CXCR4^-$ $CD4^+$ U87 glioma cells (22, 33). Also, the HIV-2 strains used have a broader tropism for $CD4^+$ human and nonhuman cell types (6, 20), several of which do not express CXCR4. HIV-2 ROD/B is a variant of the prototype ROD strain (ROD/A) that can infect certain $CD4^-$ human cell types (7) with CXCR4 alone as a receptor (13) yet still retains the broad, CD4-dependent tropism characteristic of most T-cell-line-passaged HIV-2 isolates (reference 20 and unpublished observations). Uninfected target cells (e.g., RD/CD4 cells) were treated for 30 min with 12G5 dilutions before addition of an equal number of H9 cells chronically infected with an appropriate HIV-1 or HIV-2 strain. Cocultivations were incubated at 37°C overnight before syncytium formation was estimated as described before (6, 7, 20). Table 3 shows that 12G5 inhibited induction of cell-to-cell fusion of $CXCR4^+$ RD/CD4 cells by all HIV-1 and HIV-2 strains tested. Thus, each of the seven HIV isolates tested uses CXCR4 as a coreceptor on RD/CD4 cells during fusion.

We next tested whether 12G5 could inhibit HIV-1- and HIV-2-induced cell-to-cell fusion of $CXCR4^+$ $CD4^+$ T-cell lines as well as other $CD4^+$ cell types (either $CXCR4^+$ or $CXCR4^-$). Table 3 shows that 12G5 failed to inhibit cell-to-cell induction of fusion by any of the seven viruses on the T-cell lines MOLT-4, Sup T1, and MT-2, although a slight but consistent reduction in syncytium formation was seen for the HIV-1 GUN-1 strains on MOLT-4 and Sup T1 cells with the highest dose of 12G5 (20 μg/ml). In contrast, fusion of C8166 and SSCEM T cells as well as HeLa/CD4 cells by both HIV-1 GUN-1wt and Gun-1var was blocked by 12G5 even though fusion by the other strains on these cell types was resistant. Likewise, HIV-2 CBL-23 was blocked for fusion on C8166 and HeLa/CD4 yet resisted 12G5 inhibition on SSCEM. 12G5 inhibition of HIV-1- and HIV-2-induced cell fusion of cell types other than RD/CD4 is therefore complex, being cell type as well as virus strain dependent. Clearly, some strains are efficiently inhibited while others are resistant.

At this point we had tested 12G5 inhibition of HIV-1- and HIV-2-induced cell fusion. We next tested whether infection by cell-free virus was also inhibited. RD/CD4 or HeLa/CD4 were seeded at 2×10^4 cells per well in 24-well trays 2 days before infection. Cells were treated for 30 min with 100 μl of appropriate 12G5 antibody dilutions. Virus supernatant (100 μl) containing between 50 and 100 focus-forming units was added, and the cultures were incubated for a further 90 min. Inocula were then removed, and cells were washed twice before addition of 1 ml of growth medium and incubation for 4 days. Foci of infection were detected by immunostaining as previously described (7). Figures 2A and B show that the pattern of 12G5 inhibition of infectivity for RD/CD4 cells and HeLa/CD4 cells exactly followed that observed for inhibition of cell fusion. The infectivity of all strains tested on RD/CD4 cells was inhibited by 12G5. However, on HeLa/CD4 cells, GUN-1wt was blocked by 12G5 whereas only slight inhibition of the LAI and RF strains was observed.

The simplest explanation for these results is that certain HIV strains can use coreceptors other than CXCR4 on some cell types (and particularly on $CD4^+$ T-cell lines) and are therefore not substantially inhibited by 12G5. For the T-cell-line-adapted strains used in this study, CXCR4 must be the only coreceptor choice on $CXCR4^+$ $CD4^+$ RD cells, so the anti-CXCR4 MAb 12G5 inhibits infection. On most $CXCR4^+$ $CD4^+$ T-cell lines, 12G5 failed to inhibit HIV-1 and HIV-2

TABLE 3. Inhibition of HIV-1- and HIV-2-induced syncytium formation on $CD4^+$ cell lines by 12G5

CD4+ cell line	Titer of 12G5 syncytium-inhibiting activity[a]: HIV-1 strain				HIV-2 strain	
	LAI	RF	Gun-1wt	Gun-1var	ROD A/B	CBL-23
RD/CD4	2.5	2.5	1.25	0.625	5	2.5
HeLa/CD4	>20[b]	>20[b]	5	5	>20	5
C8166	>20	>20	2.5	2.5	>20	2.5
MOLT 4	>20	>20	>20[b]	>20[b]	>20	>20
Sup T1	>20	>20	>20[b]	>20	>20	>20
MT-2	>20	>20	>20	>20	>20	>20
U87/CD4/CXCR4	>20					

[a] Antibody titers were estimated as the highest concentration of 12G5 (μg/ml) that inhibited >95% syncytium formation; i.e., >20 means there was no inhibition by 12G5 up to a concentration of 20 μg/ml, whereas 1.25 means that 12G5 blocked >95% of syncytium formation at 1.25 μg/ml but lower concentrations inhibited less or not at all. Unless otherwise noted, the titers shown refer to >95% syncytium inhibition.
[b] Consistent reduction of up to 50% syncytium formation at 20 μg/ml was seen.

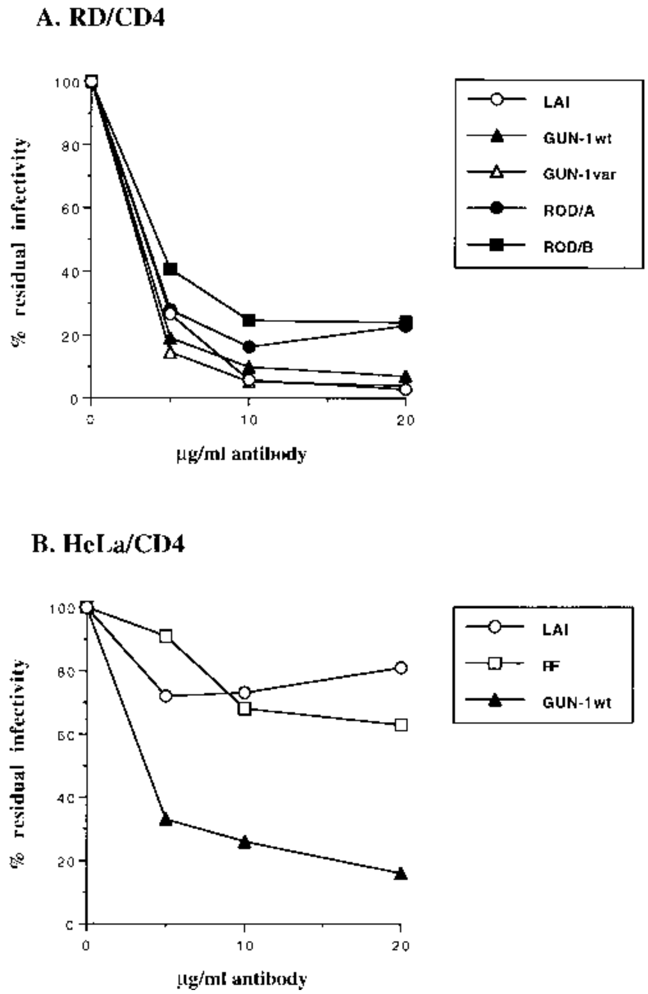

FIG. 2. 12G5 inhibition of cell-free HIV infectivity for RD/CD4 cells (A) and HeLa/CD4 cells (B). See text for details.

fusion, suggesting the presence of alternative coreceptors. This interpretation is supported by the observation that certain HIV-1 (GUN-1wt and Gun-1var) and HIV-2 strains can infect at least some $CXCR4^-$ $CD4^+$ cell types (Table 2) and therefore must use alternative coreceptors for entry into these cells. Thus, as reported already by Choe et al. (5) and Doranz et al. (11), at least some HIV strains can choose between compatible alternative coreceptors for CD4-dependent entry. Furthermore, we have recently shown that several primary dual-tropic HIV-1 isolates (as well as GUN-1wt) infected cat CCC cells expressing human CD4 as long as either CXCR4 or CCR5 was present, while a subset of the primary strains could use CCR3, CCR5, or CXCR4 (32). Yet, LAI- and RF-induced fusion of C8166, as well as GUN-1wt-induced fusion of MOLT-4 cells, which are resistant to 12G5 inhibition, also resisted inhibition by a combination of 12G5 (20 µg/ml) and RANTES (200 ng/ml). RANTES binds to a number of CC chemokine receptors (3, 25, 29, 30), including CCR5, a coreceptor for NSI macrophage-tropic HIV-1 strains (1, 10, 12, 34) and inhibits PBMC infection by NSI macrophage-tropic strains (9, 10, 12). Thus, if alternative coreceptors present on $CD4^+$ T-cell lines are exploited by the virus strains used here, it is unlikely that they are receptors for RANTES.

Yet, we favor the interpretation that the processing or presentation of CXCR4 is different on different cell types, enabling at least some HIV strains to evade 12G5 inhibition. Perhaps on some cell types, other cell surface molecules interact with CXCR4, influencing HIV recognition of the coreceptor. Indeed, expression of CXCR4 on $CD4^+$ U87 cells (10, 13) confers sensitivity to LAI fusion which is resistant to 12G5 inhibition (Table 3). Thus, at least for this situation, in which recombinant CXCR4 should be the only coreceptor available for LAI on U87/CD4 cells, 12G5 fails to block fusion. Furthermore, although 12G5 failed to block LAI infection of HeLa/CD4 cells, others have shown that the CXCR4 ligand SDF does block infection (2, 27). Assuming that SDF is specific for CXCR4, this observation implies that CXCR4 is the only coreceptor available for LAI on HeLa/CD4 cells. Thus, although 12G5 binds to CXCR4 on both RD/CD4 and HeLa/CD4 cells, differences in the processing or presentation of CXCR4 must enable LAI to resist 12G5 inhibition on HeLa/CD4 cells but not on RD/CD4 cells.

The existence of different forms of CXCR4 presentation on different cell types may explain the cell-type-dependent inhibition of HIV strains shown here but does not resolve why inhibition on some cell types (e.g., C8166 and HeLa/CD4) was also virus strain dependent. It is thus likely that different strains interact differently with CXCR4 to trigger fusion. Although the natures of the different interactions are currently unknown, this hypothesis is supported by our recent observation that deletions at the N terminus of CXCR4 have different effects depending on the HIV strain tested (27a).

In summary, we have shown that there is inhibition of HIV-induced infection and cell-to-cell fusion by a novel MAb (12G5) to CXCR4, the coreceptor for T-cell-line-adapted HIV strains. 12G5 inhibition was cell type and virus strain dependent. Variations in processing or presentation of CXCR4 or expression of alternative coreceptors meant that 12G5 failed to block most HIV-1 and HIV-2 strains on $CD4^+$ T-cell lines. These results indicate that the designing of therapeutic molecules to intervene in HIV and coreceptor interaction will not be straightforward and suggest that resistant escape mutants will emerge.

We thank Yasu Takeuchi (Chester Beatty Laboratories, London, United Kingdom), Nobu Shimizu, and Hiroo Hoshino (both at Gunma University Medical School, Maebashi, Japan), who kindly provided HIV-1 GUN-1 viruses, and Ned Landau, who provided CXCR4-expressing U87/CD4 cells. Thanks to Robin Weiss for critical discussion and reading of the manuscript, to Ian Titley for advice on flow cytometry, and to Matthias Dittmar, Jackie Reeves, and Sandra Potempa for valuable discussion.

Our work is supported by the Medical Research Council.

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